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ORIGINAL ARTICLE

Raw starch–degrading α-amylase from *Bacillus aquimaris* MKSC 6.2: isolation and expression of the gene, bioinformatics and biochemical characterization of the recombinant enzyme

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Keywords
B. aquimaris MKSC 6.2, GH13 subfamily, raw starch degrading, α-amylase.

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Dedicated to the memory of Achmad Saifudin Noer who passed away in 2010.

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Abstract

Aims: The aims were to isolate a raw starch–degrading α-amylase gene *baqA* from *Bacillus aquimaris* MKSC 6.2, and to characterize the gene product through *in silico* study and its expression in *Escherichia coli*.
Methods and Results: A 1539 complete open reading frame of a starch–degrading α-amylase gene *baqA from B. aquimaris* MKSC 6·2 has been determined by employing PCR and inverse PCR techniques. Bioinformatics analysis revealed that *B. aquimaris* MKSC 6.2 α-amylase (BaqA) has no starch-binding domain, and together with a few putative α-amylases from bacilli may establish a novel GH13 subfamily most closely related to GH13_1. Two consecutive tryptophans (Trp201 and Trp202, BaqA numbering) were identified as a sequence fingerprint of this novel GH13 subfamily. *Escherichia coli* cells produced the recombinant BaqA protein as inclusion bodies. The refolded recombinant BaqA protein degraded raw cassava and corn starches, but exhibited no activity with soluble starch.
Conclusions: A novel raw starch–degrading *B. aquimaris* MKSC 6.2 α-amylase BaqA is proposed to be a member of new GH13 subfamily.
Significance and Impact of the Study: This study has contributed to the overall knowledge and understanding of amylolytic enzymes that are able to bind and digest raw starch directly.

Introduction

Starch-degrading enzymes cleave glycosidic bonds in starch either from the nonreducing end (exo-acting enzymes) or in the interior (endo-acting enzymes) of the polymer. α-Amylases (EC 3.2.1.1) are endo-acting enzymes cleaving α-1,4-glycosidic bonds in starch to form various maltooligosaccharides and maltodextrin (MacGregor *et al.* 2001; van der Maarel *et al.* 2002).

α-Amylases are mainly classified into glycoside hydrolase (GH) family 13 (Henrissat 1991). The GH13 family covers enzymes with several reaction specificities, but they share common conserved sequence regions (CSRs) with a catalytic triad (Asp, Glu, Asp), employ an α-retaining mechanism, and adopt a Triosephosphate IsoMerase (TIM)-barrel fold (Janecek 2002). A few years ago, Stam *et al.* (2006) divided 1691 different members of the GH13 family into 35 subfamilies based on their amino

acid sequence similarities. Most subfamilies are monofunctional, while the rest contain polyspecific enzymes with strongly related substrate and/or product specificities. Nowadays (August 2012), 37 subfamilies from more than 12 000 different members of this family can be found at the CAZy website (http://www.cazy.org/), that is, a database specialized in classifying the carbohydrate-active enzymes that build and degrade complex carbohydrates (Cantarel *et al.* 2009). Bacterial α-amylases are classified into GH13 subfamilies 5, 27, 28, 32, 36 and 37. Subfamily GH13_36 contains the so-called intermediary enzymes that exhibit a mixed enzyme specificity of α-amylase and an additional GH13 specificity (Oslancova and Janecek 2002). Various novel putative α-amylases identified in genome projects have not been classified yet into any subfamily (Cantarel *et al.* 2009).

α-Amylases from microbes are applied widely in the starch industry and in food processing (Gupta *et al.* 2003). Bacteria from the genus *Bacillus* are well known as α-amylase producers. Some bacilli were reported to possess raw starch–degrading enzyme activities (Demirkan *et al.* 2005; Goyal *et al.* 2005; Mitsuiki *et al.* 2005). A marine *Bacillus* associated with soft coral *Sinularia* sp., *Bacillus aquimaris* MKSC 6.2, also produced raw starch–degrading amylolytic enzymes (Puspasari *et al.* 2011). Raw starch–degrading amylases are of great interest in view of their ability to hydrolyze raw starch at moderate temperature, which is the key to reduce production cost in starch-processing industry (Leveque *et al.* 2000). Most raw starch–degrading enzymes possess a starch-binding domain (SBD) responsible for binding and raw starch degradation (Machovic and Janecek 2006; Janecek *et al.* 2011), localized usually at the C-terminus of bacterial and fungal amylases (Janecek and Sevcik 1999). Such a distinct SBD is present in approximately 10% of all amylolytic enzymes (Machovic and Janecek 2006), but amylases without such a specialized domain able to bind and digest raw starch also exist (Søgaard *et al.* 1993; Hostinova *et al.* 2003). Usually tryptophan (or in a wider sense aromatic) residues are responsible for these so-called surface-binding sites (Gibson and Svensson 1987; Gyemant *et al.* 2009) that, for example, in the barley α-amylase isozyme AMY1 and *Saccharomycopsis fibuligera* glucoamylase are arranged as so-called 'sugar tongs', that is, a sugar molecule entrapped by a tyrosine (Robert *et al.* 2003; Sevcik *et al.* 2006).

In this study, we report the nucleotide sequence of the *B. aquimaris* MKSC 6.

Two α-amylase gene (*baqA*), its expression in *Escherichia coli* and bioinformatics analysis together with biochemical characterization of the recombinant enzyme. Sequence and phylogenetic analyses of BaqA and some other novel putative α-amylases from genus *Bacillus* revealed that these α-amylases, grouped separately in a novel cluster in the evolutionary tree, may define a new GH13 subfamily.

Materials and methods

Strains and plasmids

Bacillus aquimaris MKSC 6.2 (*Bacaq*) was obtained from the culture collection at the Center for Tropical Coastal and Marine Studies, Diponegoro University, Semarang, Indonesia. The bacterium was isolated from a soft coral *Sinularia* sp. obtained from the sea around Merak Kecil Island, Banten, Indonesia. *Escherichia coli* strain TOP10F′ (Invitrogen) was used for gene manipulation. The host strain for heterologous recombinant expression was *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany). Plasmid pGEM-T (Promega, Madison, WI) was used to clone PCR products, while pET30a (+) (Novagen) was used as an expression vector.

Isolation of the α-amylase gene

Isolation of *Bacaq* α-amylase gene (*baqA*) was conducted through several PCR steps using degenerate primers (degPCR) and inverse PCR (invPCR) (Weber-Arden *et al.* 1996). The nucleotide sequences of degenerate primers were 5′-GAYTTYRTYGTSAATCAYGTYGG-3′ (degFA), 5′-GATGGRTAYCGTCTRGATACYG-3′ (degFB), 5′-AATCGWMYCATATCATGGTTATC-3′ (degR2) and 5′-CAGATCCRTAGTAAASAATSGG-3′ (degR4). The first invPCR used primers InvF1 5′-TGACCCACAGAAGATAGCAG-3′ and InvR1 5′-ATGGCGCACAGTATCCAGAC-3′ with circularized *Cla*I cut *Bacaq* chromosomal DNA as template. The second invPCR was performed with circularized *Hin*dIII cut *Bacaq* chromosomal DNA as template using InvF2 5′-TACACGACTCCGGGAATACC-3′ and InvR1. The last one used primers InvF3 5′-AGCGGGATATGAAAGTCGTC-3′ and InvR3 5′-GAGTCAGCCATATGGAAGTG-3′ with circularized *Hpa*I cut *Bacaq* chromosomal DNA as template. Purified PCR products were cloned into plasmid pGEM-T and sequenced through dideoxy Sanger method using dye terminator (Macrogen, Seoul, Korea). The nucleotide sequence of *baqA* has been deposited in Genetic sequence database at the National Center for Biotechnical Information (NCBI) (GenBank ID: JN797599).

Cloning of *baqA* gene

The whole *baqA* gene without putative signal peptide encoding region was amplified by PCR using primers baqA-F 5′-GATATCGAAGAACGAAAGTGGCAG-3′ and

baqA-R 5′-GAATTCGATTTGCGGTTTTTTCTTCCG-3′. Forward and reverse primers carried *Eco*RI and *Eco*RV restriction sites, respectively. The amplified gene was cloned in pGEM-T (Promega), and the sequence was verified. It was then subcloned into pET30a(+) (Novagen) to construct the recombinant plasmid pET30-baqA.

Production and refolding of recombinant BaqA

Escherichia coli BL21 (DE3) carrying pET30-baqA was grown in 50-ml LB medium [0·5% (w/v) yeast extract; 1% (w/v) tryptone; 0·5% (b/v) NaCl] containing 30 μg ml^{-1} kanamycin in a rotary shaker (37°C, 150 rev min^{-1}) to an optical density of 0·4–0·6 (600 nm). Expression of recombinant *baqA* was induced by 0·5 mmol l^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) 37°C for 4 h and analyzed by SDS-PAGE. Cells were harvested by centrifugation (13 000 ***g***, 30 min) and then disrupted by sonication. Inclusion bodies were pelleted by centrifugation (13 000 ***g***, 10 min) and dissolved by addition of sarkosyl [final concentration was 0·3% (w/v)] as described by Marshak *et al.* (1996). After centrifugation (13 000 ***g***, 10 min), the solubilized protein was diluted tenfold with 20 mmol l^{-1} maleate buffer (pH 6·5) and dialyzed against 20 mmol l^{-1} maleate buffer (pH 6·5) for 20–24 h to remove the detergent. Protein was subjected to SDS-PAGE (Laemmli 1970). This refolded recombinant BaqA α-amylase was used for biochemical studies.

α-Amylase activity toward soluble starch

α-Amylase activity was determined by measuring the amount of reducing sugars formed using a modification of the dinitrosalicylic acid (DNS) method (Miller 1959). The reaction was performed in a reaction mixture containing 25 μl of 1% soluble starch (E-Merck, Darmstadt, Germany) and various amounts of enzyme in 50 mmol l^{-1} maleate buffer (pH 6·5) at 37 and 50°C for various time intervals up to 24 h. The reaction was stopped by addition of 50 μl of DNS solution and incubated in boiling water bath for 10 min. The reaction mixture was cooled down to room temperature, and the absorbance at 500 nm was measured. The amount of reducing sugar was then determined using a glucose standard curve. One unit of amylase activity equals the amount of the enzyme producing 1 μmol of reducing sugars per min under the assay conditions. All assays were performed in triplicates.

Raw starch digestion

To determine the digestion of raw starch by recombinant BaqA, reaction mixtures containing 50 μg of the enzyme and 10% (w/v) raw starch (corn or cassava) to a final volume of 0·2 ml in 50 mmol l^{-1} universal buffer (pH 6·5) were incubated at 37°C with shaking for 24 h. After centrifugation, the reducing sugar produced in supernatant was determined using a modification of the DNS method. The degree of hydrolysis (DH) was calculated by the following equation: DH (%) = (H_1/H_0) × 100, where H_1 was reducing sugar produced by enzyme hydrolysis, and H_0 was reducing sugar produced by acid hydrolysis. Acid hydrolysis was carried out by treating raw starch with 1 mol l^{-1} HCl at 100°C for 2 h (Wang *et al.* 1995).

Raw starch adsorbability

Affinity of the enzyme towards raw corn and cassava starch was studied by incubating 24 μg of the enzyme with 5–350 mg of raw starch at 4°C for 1 h. After centrifugation, the amount of free enzyme in the supernatant was determined. The bound protein was the difference between original protein amount and the free protein amount in the supernatant after binding. Total protein concentration was determined by Bradford method (Bradford 1976) using bovine serum albumin as a standard.

Scanning electron microscopy and end products determination

A mixture of 1% (w/v) of raw corn or cassava starch and 800 mg of recombinant BaqA to a final volume of 0·2 ml in 50 mmol l^{-1} maleate buffer (pH 6·5) was incubated at 37°C for 48 h. After centrifugation, the pellet was washed with 95% ethanol and then dried at 35°C. The treated starch granules were coated with Au-Pd (80–20) using Ion Sputter JFC-110 at 1·2 KV and 6 mA for 4 min and photographed using scanning electron microscopy (SEM) (JSM-35C). Supernatant was tested for end products determination. End product of raw starch degradation by recombinant BaqA treatment was analyzed by thin layer chromatography using a stationary phase of 10-cm silica gel plate (E-Merck) and a solvent system containing butanol:ethanol:water (5:5:3). End product spots were visualized by spraying the plate with 50% (v/v) of sulphuric acid in methanol and heated at 110°C for 10 min.

Bioinformatics

Nucleotide sequences of PCR products were used as the query sequence in the BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTX program (Altschul *et al.* 1997). The type of family and domain in the deduced protein were searched against the Pfam database (http://pfam.sanger.ac.uk/; Finn *et al.* 2010). Prediction of

a signal peptidase cleavage site was performed by the web-based search tools SignalP (http://www.cbs.dtu.dk/services/; Bendtsen *et al.* 2004).

The amino acid sequence of BaqA deduced from the nucleotide sequence was compared with those of other α-amylases retrieved from the Universal Protein Resources Knowledgebase (UniProt; Apweiler *et al.* 2011) and GenBank (Benson *et al.* 2011) databases. The set covered all GH13 subfamilies (Stam *et al.* 2006; Cantarel *et al.* 2009) with well-defined (GH13 subfamilies 1, 5, 6, 7, 15, 24, 27, 28, 32 and 37) and supposed (GH13_36) α-amylase activity (EC 3.2.1.1) completed with representatives of the bacterial subfamily GH13_19 (closely related sequences with mostly maltohexaose-forming amylase specificity). The aligned amino acid sequence segments spanned the region (approximately 300 residues) of the catalytic $(\beta/\alpha)_8$-barrel from β1 to β8 strands including domain B (Da Lage *et al.* 2004; Hostinova *et al.* 2010) and containing all the seven CSRs of the α-amylase family (Janecek 2002). The alignment was performed using the ClustalX program (Jeanmougin *et al.* 1998), and the evolutionary tree was calculated from the manually adjusted alignment as a Phylip-tree type with the neighbour-joining clustering method (Saitou and Nei 1987) and the bootstrapping procedure (Felsenstein 1985; the number of bootstrap trials used was 1000) implemented in the ClustalX package. The tree was displayed with the TreeView program (Page 1996).

A BaqA three-dimensional structure model was obtained with the Phyre (Protein Homology/AnalogY Recognition Engine) fold recognition server (http://www.sbg.bio.ic.ac.uk/phyre/; Kelley and Sternberg 2009). The structure of the BaqA model was compared with the experimentally determined structure of barley α-amylase low pI isozyme AMY1, a raw starch–degrading α-amylase without an SBD (Robert *et al.* 2003), and that of *Aspergillus niger* α-amylase solved in the complex with a maltose molecule bound outside the active site (Vujicic-Zagar and Dijkstra 2006). The structures of these barley and *Aspergillus* α-amylases were retrieved from the Protein Data bank (PDB; Rose *et al.* 2011) under the codes 1P6W and 2GVY, respectively. The structures were overlapped with the program MultiProt (http://bioinfo3d.cs.tau.ac.il/MultiProt/; Shatsky *et al.* 2004).

Results

Nucleotide sequence of *baqA*

The combined nucleotide sequences from all degPCR and invPCR products resulted in an open reading frame (ORF) of *baqA* consisting of 1539 bp. The BaqA polypeptide deduced was 512 amino acid residues in length, including the first 23 residues that formed a putative signal peptide (Fig. 1). Seven CSRs of the GH13 α-amylase family (Janecek 2002) and the catalytic triad (MacGregor *et al.* 2001) were identified in BaqA. The catalytic triad consists of Asp214 (catalytic nucleophile at β4), Glu243 (proton donor at β5) and Asp311 (transition-state stabilizer at β7).

To characterize the protein encoded by *baqA*, the *baqA* fragment encoding for 27–512 amino acid residues was PCR amplified, cloned into pET30 vector and then expressed in *Escherichia coli* BL21 (DE3). Intracellular production of BaqA in *E. coli* resulted in the formation of inclusion bodies (Fig. 2, lane 3) which required solubilization and refolding to generate an active recombinant BaqA. The *baqA* expression cassette encoded for 553 amino acid residues consisting of BaqA without its signal sequences and N and C terminal His-tag fusions. SDS-PAGE analysis showed that the mobility of recombinant BaqA was *c.* 70 kDa, which is somewhat higher than the expected 64·4 kDa. This appears to reflect an aberrant electrophoretic mobility of recombinant BaqA on the SDS/PAGE gel.

Biochemical properties of recombinant BaqA

Recombinant BaqA could not degrade soluble starch. No detectable degradation of soluble starch was measured in all variation of enzyme amount (up to 150 μg of enzyme) nor variation of time intervals at 37 and 50°C. The refolded recombinant BaqA can degrade raw cassava and corn starch. At the experiment conditions, recombinant BaqA degraded raw cassava starch and produced 8 mmol l^{-1} reducing-end sugars; the observation which was proportional to 1·5% DH. Raw corn starch was degraded to produce 7·5 mmol l^{-1} reducing-end sugars (proportional to 1·4% DH).

The ability of recombinant BaqA to hydrolyze raw starch was confirmed by SEM. Recombinant BaqA attacked raw corn starch with result of forming the holes in starch granule surface, while it peeled away the surface of raw cassava starch (Fig. 3).

The end products of recombinant BaqA action on raw corn starch were different from those on raw cassava starch. Degradation of raw cassava starch by recombinant BaqA released glucose, maltose, maltotriose and maltotetraose, while degrading raw corn starch, only maltose, maltotriose and maltotetraose were released (Fig. 4).

Adsorption of recombinant BaqA towards raw corn and cassava starch was assayed at various amount of starch. The slope of the curve on bound protein (μg) vs. raw starch (mg) graphic indicated the amount of recombinant BaqA bound per mg raw starch. The curve would reach a plateau when all protein was bound to raw starch (100% bound protein). Figure 5 shows that 0·06 μg BaqA was absorbed to 1 mg raw corn starch. Despite their

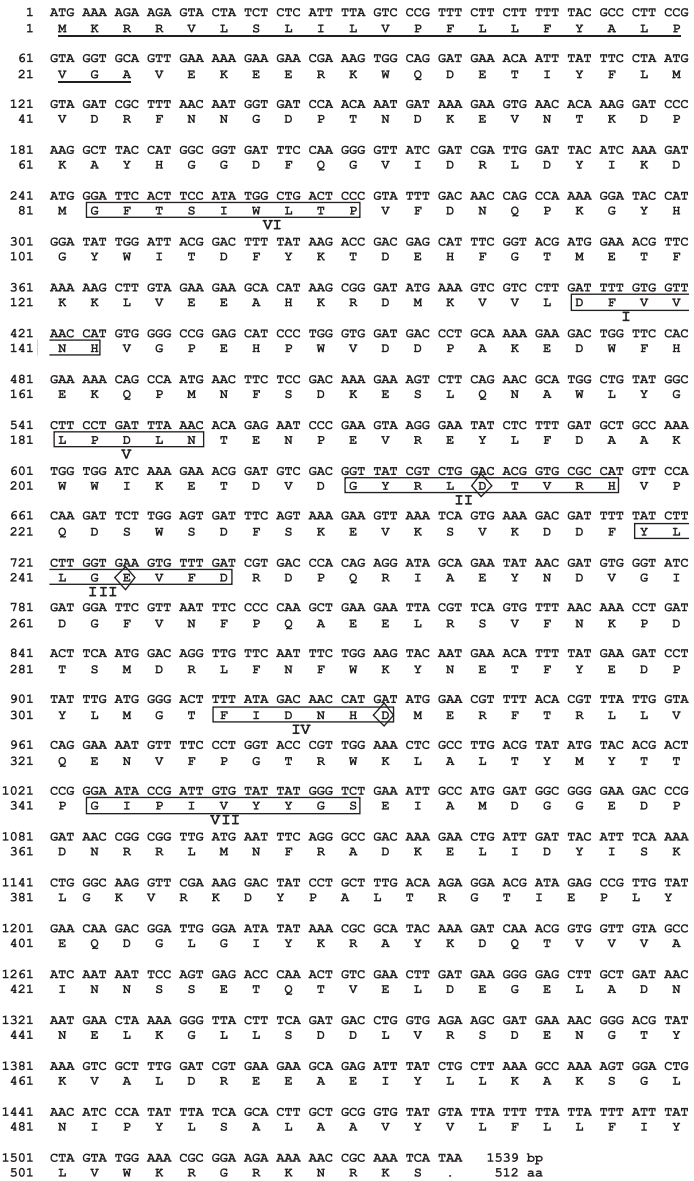

Figure 1 Nucleotide sequence of the open reading frame encoding the BaqA precursor and its deduced amino acid sequence. The putative signal peptide sequence is underlined. The seven conserved sequence regions of GH13 α-amylase family (Janecek 2002) are boxed. Catalytic triad is signified by diamonds.

equal degradation on both raw cassava and raw corn starches, the enzyme was absorbed in an amount of 0·006 μg per 1 mg of cassava starch.

Bioinformatics

The amino acid sequence of BaqA was aligned with 32 α-amylases from bacteria, archaea, fungi, plants and animals that have already been classified into various GH13 subfamilies and three additional, currently unclassified, hypothetical α-amylases. The three unclassified α-amylases originating from bacilli (*Bacillus* sp. SG-1, *Bacillus* sp. NRRL B-14911 and *Bacillus coahuilensis*) were caught by BLAST and exhibited a high degree of similarity with BaqA (data not shown). Phylogenetic tree of compared 36 sequences clearly showed the presence of a new cluster consisting of BaqA and the three unclassified α-amylases (Fig. 6) that may establish a novel GH13 subfamily. The α-amylases from the new cluster (and putative new GH13 subfamily) exhibit a specific sequence feature, that is, two consecutive tryptophan residues (Trp201 and Trp202, BaqA numbering), positioned probably at helix α3 preceding strand β4 covering the catalytic nucleophile Asp214 (Fig. 7).

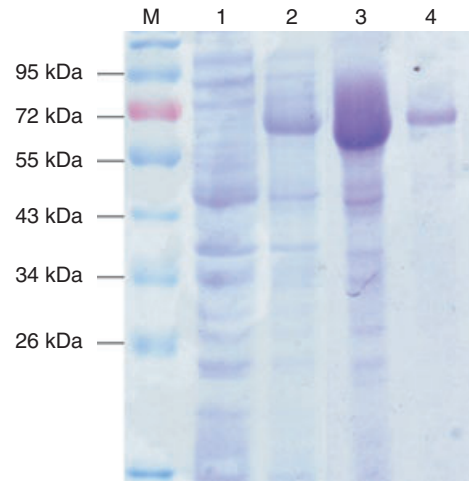

Figure 2 SDS-PAGE of recombinant BaqA. Lane M, molecular weight standard; lane 1, non-isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cell lysate; lane 2, IPTG-induced cell lysate; lane 3, inclusion bodies; lane 4, refolded recombinant BaqA.

The three-dimensional structural model obtained from the PHYRE server confirmed the expected overall fold of BaqA consisting of catalytic α-amylase-type $(\beta/\alpha)_8$-barrel (domain A) with a small domain B (located between the strand β3 and helix α3 of the barrel), succeeded by the C-terminal β-sandwich domain C (Fig. 8), that is, the domain arrangement typical for the GH13 α-amylase family members (MacGregor *et al.* 2001). The overlap of the BaqA modelled structure with the experimentally determined structures of either barley or *Aspergillus niger* α-amylases did not reveal any correspondences with the so-called sugar tongs-like starch-binding site characteristic for the plant enzyme as well as the site where a maltose molecule bound outside the active site of fungal enzyme was observed (Fig. 8).

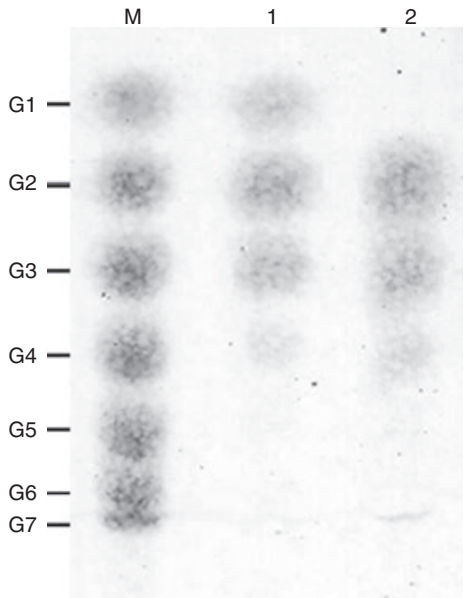

Figure 4 Thin layer chromatogram of raw starch hydrolysis products by recombinant BaqA. Lane M, G1–G7 standards (maltooligosaccharides from glucose G1 to maltoheptaose G7); lane 1, end products of raw cassava starch; lane 2, end products of raw corn starch.

Discussion

The nucleotide sequence of *Bacillus aquimaris* α-amylase gene has not been reported previously. The only genomic information of *B. aquimaris* was the nucleotide sequence of its 16S rRNA (Yoon *et al.* 2003; Puspasari *et al.* 2011).

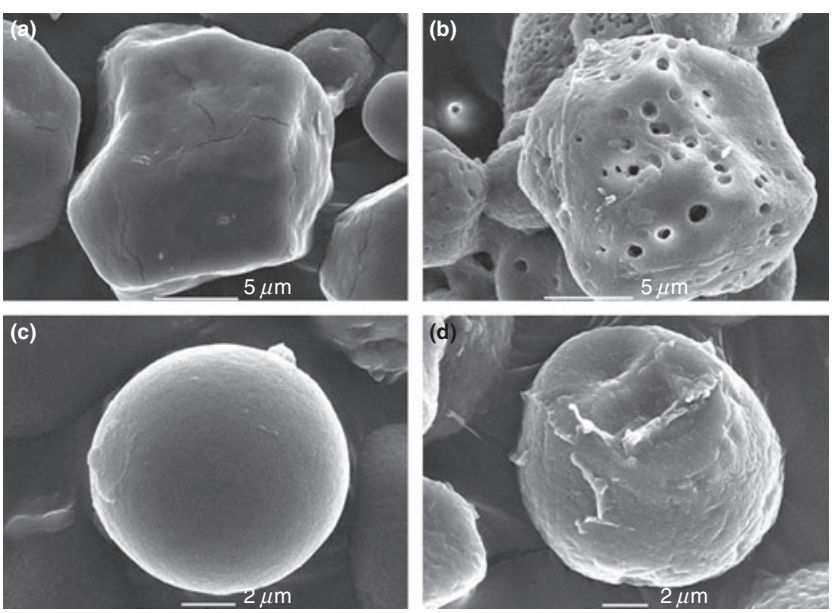

Figure 3 Scanning electron microscopy of untreated and treated raw starch granules by recombinant BaqA. (a) Untreated corn starch, (b) treated corn starch, (c) untreated cassava starch, (d) treated cassava starch.

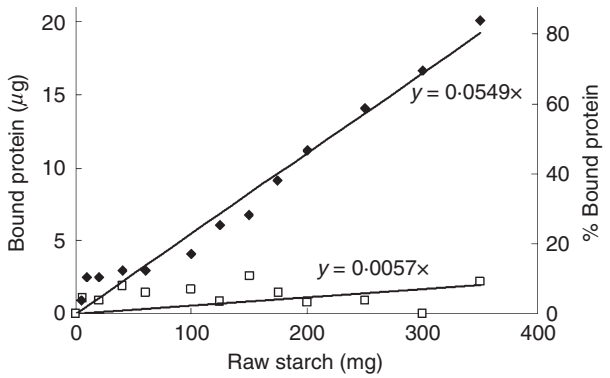

Figure 5 Adsorption of recombinant BaqA on raw corn (♦) and cassava (□) starch. The slope indicates the amount of recombinant BaqA bound per mg raw starch.

A first attempt to clone the α-amylase gene of *B. aquimaris* MKSC 6.2 employedits partial genomic library; however, the gene could not be pulled out (F. Puspasari and D. Natalia, unpublished data). Nevertheless, BLASTX analysis of the nucleotide sequences obtained from several clones of the partial genomic library revealed that some proteins of *Bacaq* exhibited the highest degree of sequence similarity (data not shown) with their counterparts from *Bacillus* sp. SG-1, *B. coahuilensis* and *Bacillus weihenstephanensis*. The degenerate primers for degPCR were then designed based on the alignment of putative α-amylase genes of the three above-mentioned bacilli. The primer degFA covered the CSR I, while primers degFB, degR2 and degR4 covered consecutively on CSRs II, IV and VII; conserved regions as proposed by Janecek (2002).

Further nucleotide sequence analysis upstream of the *baqA* ORF showed that the α-amylase gene was part of an operon together with at least one other gene encoding maltosaccharide ABC transporter, permease gene upstream with a 37-nucleotide long gap (data not shown). The polypeptide chain of BaqA exhibited the highest identity (66%) with putative α-amylases from *Bacillus* sp. NRRL B-14911 (UniProt ID: A6CT23; without gaps) and *Bacillus* sp. SG-1 (UniProt ID: Q2B943; with 7% of gaps). No distinct SBD sequence was found within BaqA (Fig. 1).

The recombinant BaqA protein expressed in *Escherichia coli* BL21 (DE3) was produced in inclusion bodies, which upon refolding showed ability to degrade raw corn and cassava starch; however, it was inactive toward soluble starch. Most raw starch–degrading enzymes degrade soluble starch as well, however one of three amylases of Poplar (*Populus canadensis*) wood lacked activity with soluble starch, instead it attacked starch granules with product characteristics of endoamylase (Witt and Sauter 1995). With our current knowledge, it is difficult to explain this property of BaqA because the amino acid sequence and structure of this Poplar wood amylase have not been reported yet. Speculatively, the presence of N and C terminal His tags may negatively affect accessibility of soluble starch into the BaqA active site and thus its activity. In future work, it will be of interest to generate a recombinant BaqA without His tags, and/or to express it as an extracellular protein in another expression system, such as *Bacillus megaterium*. Other approaches, for example, coexpression of BaqA with a chaperone to improve its solubility, could also be considered. Nevertheless, our results clearly show that the refolding strategy employed resulted in soluble recombinant BaqA competent to interact with and then to digest the raw starch.

Scanning electron microscope analysis showed that the recombinant BaqA action on raw corn starch introduced holes in the granule surface, while it caused surface peeling of raw cassava starch (Fig. 3). The same pattern of hydrolysis toward raw corn and cassava starch was also observed on partially purified nonrecombinant *Bacaq* α-amylase (Puspasari *et al.* 2011) and some other α-amylases from marine *Bacillus* (Vidilaseris *et al.* 2009; Nurachman *et al.* 2010). Recently, Sarian *et al.* (2012) reported characteristics of a *Microbacterium aurum* strain B8.A α-amylase attacking a range of starch granules from diverse plant sources by initially introducing holes, followed by complete degradation. Furthermore, a new subfamily GH13_37 α-amylase from a marine metagenomic library showed a preferential raw rice starch degradation forming deep holes in the granule surface (Lei *et al.* 2012). These various enzyme mechanisms for granule degradation maybe correlated with differences in crystallinity and morphology of the various plant granules used.

Raw cassava starch hydrolyzed by recombinant BaqA yielded glucose, maltose, maltotriose and maltotetraose, while hydrolysis of raw corn starch yielded the same oligosaccharides but without glucose (Fig. 4). These results indicated that recombinant BaqA is a saccharifying or liquefying α-amylase depending on the type of raw starch used as substrate.

The adsorbability of recombinant BaqA (Fig. 5) on raw starch was found to be relatively low, for example, compared with that of raw starch–degrading α-amylase of *Lactobacillus amylovorus* (an α-amylase having a SBD), in which 30-μg α-amylase was absorbed per 1-mg raw corn starch (Rodriguez-Sanoja *et al.* 2000). On the other hand, α-amylase from yeast *Saccharomycopsis fibuligera* showed raw starch–degrading activity but no enzyme adsorption onto raw starch (Hasan *et al.* 2008; Hostinova *et al.* 2010); it is important to note that the α-amylase of *S. fibuligera* does not contain any SBD. It, thus, appears that the presence of SBD is not essential for degradation of raw starch by an amylase, but it obviously results in better enzyme adsorption onto raw starch (Christiansen *et al.* 2009).

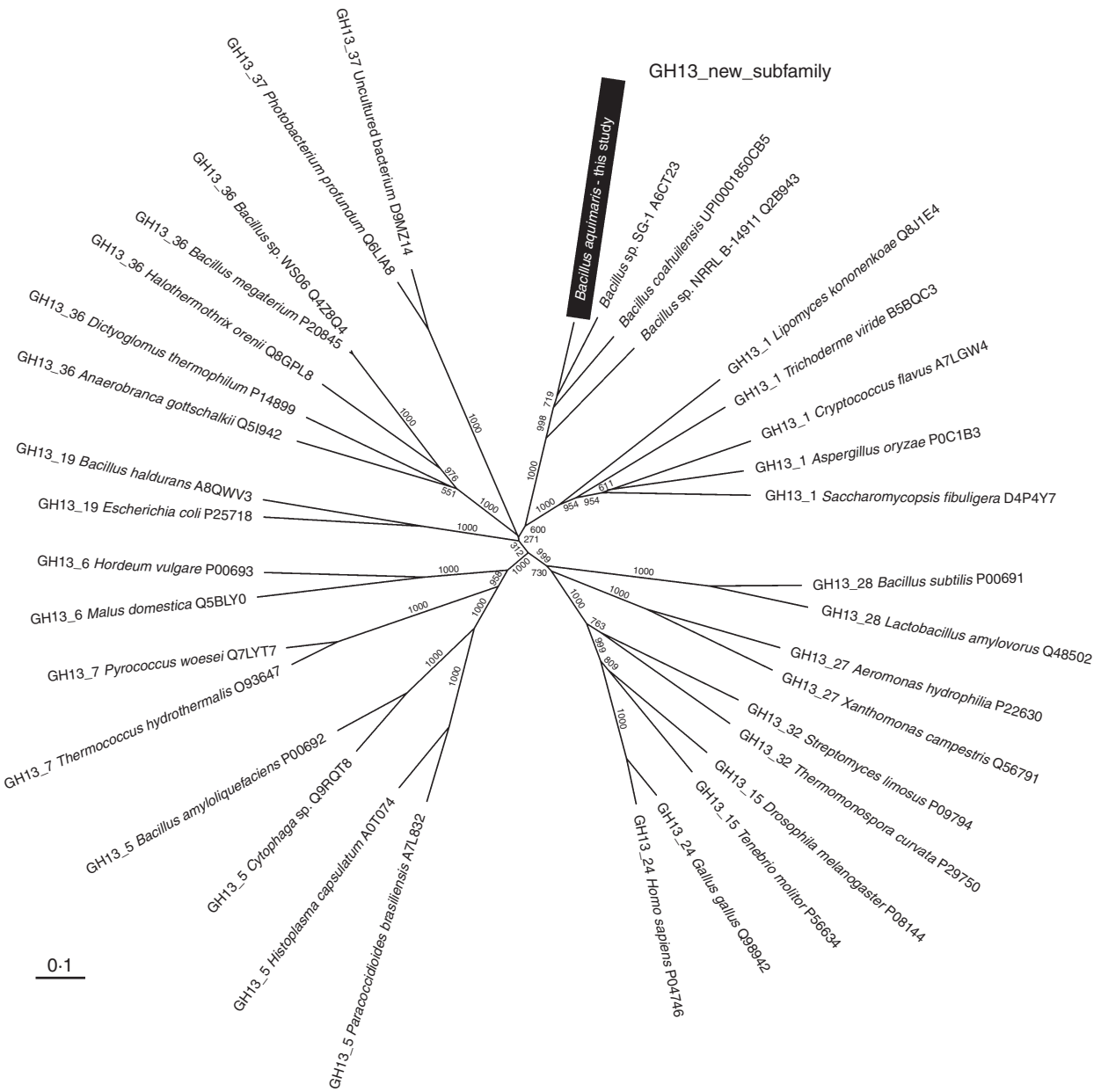

Figure 6 Evolutionary tree of the family GH13 α-amylases. α-Amylase from *Bacillus aquimaris* MKSC 6.2 and some other α-amylases from bacilli, grouped in a novel cluster separated from other previously known GH13 subfamilies, can form a novel subfamily. The individual α-amylases are represented by the GH13 subfamily number, the binomial names of their producers and the UniProt accession numbers (except for that from the *Bacillus coahuilensis*, for which the UniParc archive number is used because the UniProt one is still not available).

The phylogenetic analysis of 36 α-amylase (or, in a few cases, putative α-amylase) amino acid sequences has shown that BaqA groups with three currently unclassified hypothetical α-amylases from *Bacillus* sp. SG-1, *B. coahuilensis* and *Bacillus* sp. NRRL B-14911 (Fig. 6). The new cluster may constitute a novel GH13 subfamily (Stam *et al.* 2006) within the CAZy database (Cantarel *et al.* 2009). The novel GH13 subfamily cluster is most closely related to that of extracellular fungal α-amylases (GH13_1 subfamily), represented here by α-amylases from *Lipomyces kononenkoae* (Eksteen *et al.* 2003), *Trichoderma viridae* (Noguchi *et al.* 2008), *Crytococcus flavus* (Galdino *et al.* 2008), *S. fibuligera* (Hostinova *et al.* 2010) and the well-known Taka-amylase from *Aspergillus oryzae* (Matsuura *et al.* 1984). Other fungal (intracellular) α-amylases form the GH13_5 subfamily together with some bacterial α-amylases (van der Kaaij *et al.* 2007), but in the phylogenetic tree, these are clearly separate from

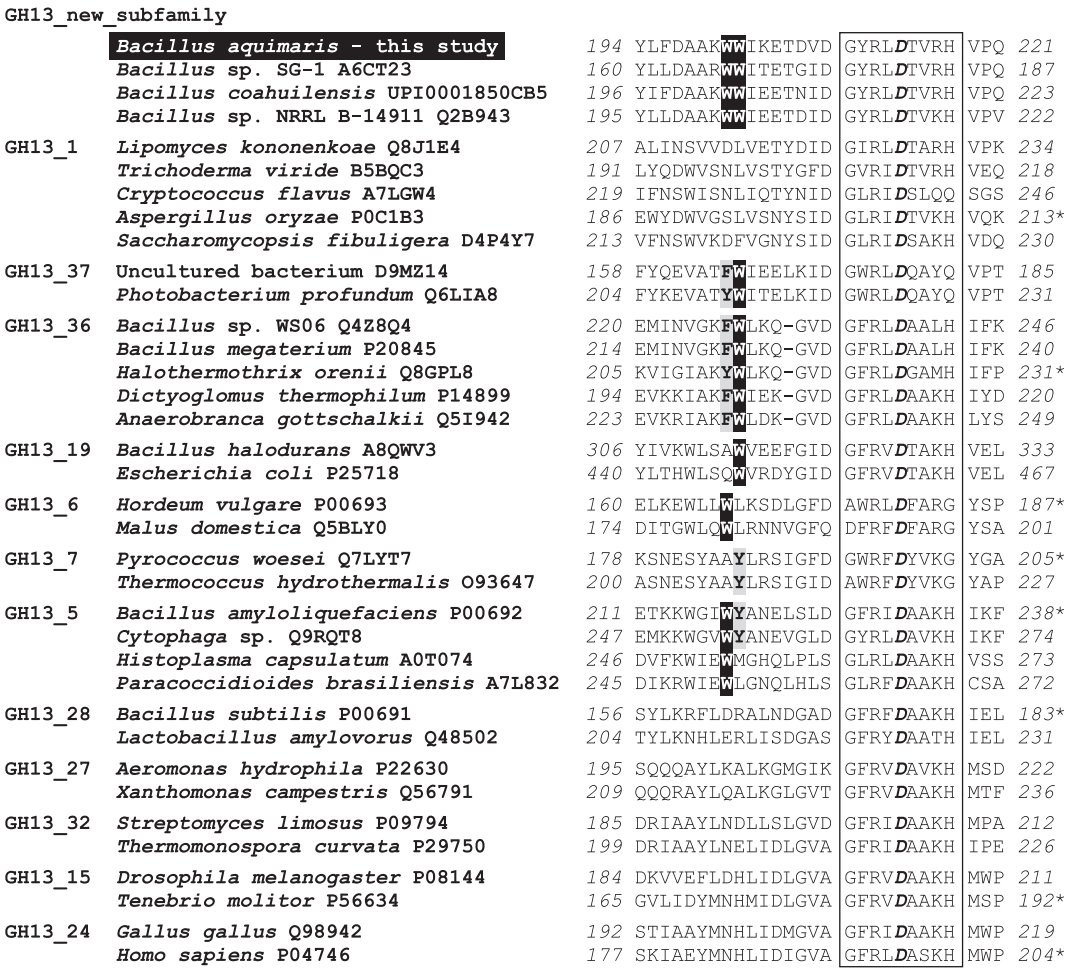

Figure 7 The new GH13 subfamily can be characterized by a sequence fingerprint of two consecutive tryptophan residues (highlighted in black). The corresponding aromatic residues from the α-amylases of other subfamilies – phenylalanines and tyrosines are both coloured grey. The catalytic nucleophile (Asp 214 in *Bacillus aquimaris* α-amylase) located in the conserved sequence region II (boxed) covering the strand *β*4 is bolded and italicized. The sequences marked with the asterisk at the end are numbered as mature proteins (i.e. without signal peptide). The individual α-amylases are represented by the GH13 subfamily number, the binomial names of their producers and the UniProt accession numbers (except for that from the *Bacillus coahuilensis*, for which the UniParc archive number is used because the UniProt one is still not available).

the new cluster, on a branch leading also to related plant and archaeal α-amylases (Janecek *et al.* 1999; Godany *et al.* 2010). It is worth mentioning that the subfamilies GH13_19 and GH13_36 may lack 'true' α-amylase specificity because the enzyme from *Bacillus halodurans* (GH13_19; Murakami *et al.* 2007) possesses very probably maltohexahydrolase specificity (Boraston *et al.* 2006), whereas the enzyme from *Halothermothrix orenii* (GH13_36; Mijts and Patel 2002) represents a large group of amylolytic enzymes with a mixed enzyme specificity and 'intermediary' character between oligo-1,6-glucosidase and neopullulanase subfamilies (Oslancova and Janecek 2002). Both these subfamilies were included in the present analysis due to their close relatedness to the new cluster (and putative novel GH13 subfamily) represented by the α-amylase from *B. aquimaris*. Interestingly, also the recently established α-amylase subfamily GH13_37 (http://www.cazy.org/) represented by a raw starch–degrading α-amylase from an uncultured bacterium (Lei *et al.* 2012; Liu *et al.* 2012) clusters separately (Fig. 6) from the emerging BaqA GH13 subfamily. The cluster of sequences represented by the *B. aquimaris* α-amylase thus may constitute a novel independent GH13 subfamily. The remaining five GH13 subfamilies with α-amylase reaction specificity originating from various distinct taxonomic groups (GH13_15, 24, 27, 28 and 32) were included in the comparison in order to complete the current α-amylase evolutionary picture (Fig. 6).

Based on a detailed comparison of amino acid sequences, it is suggested that the two consecutive tryptophan residues (Trp201 and Trp202, BaqA numbering) located at the helix α3 that precedes the strand *β*4 (the

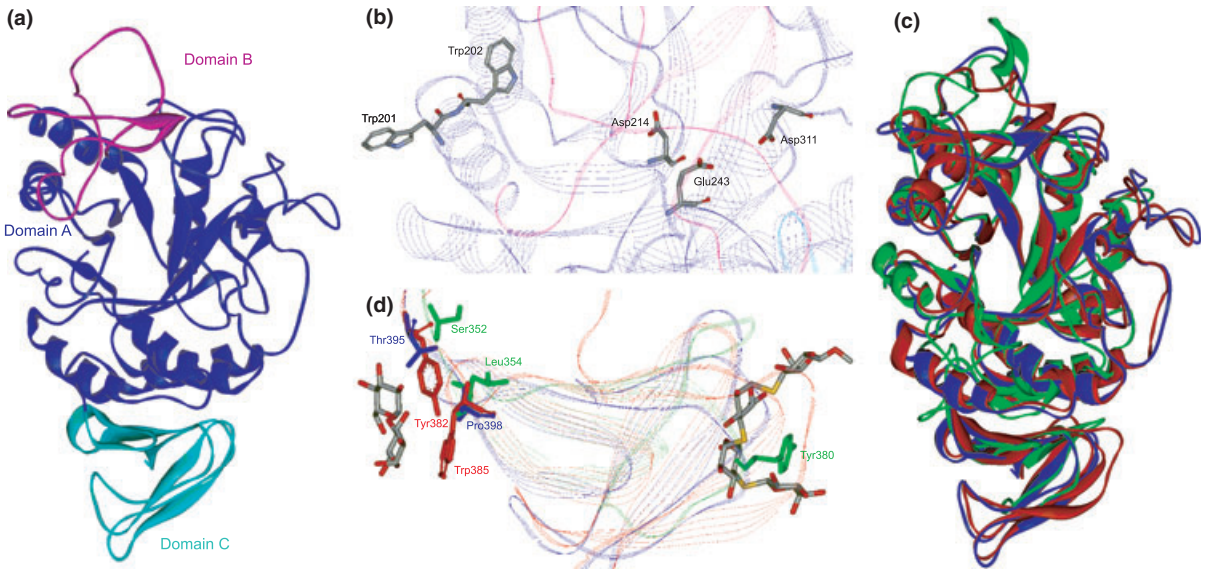

Figure 8 (a) Overall fold of the three-dimensional structure model of *Bacillus aquimaris* α-amylase. The enzyme consists of three domains A (catalytic TIM-barrel; blue), B (magenta) and C (cyan), characteristic of the α-amylase family GH13, without a distinct SBD. (b) The side chains of two consecutive tryptophans (Trp201 and Trp202) positioned on the helix α3 of domain A are shown to be oriented outside the space occupied by the catalytic triad. (c) Overlap of the BaqA model (blue) with the GH13_1 α-amylase from *Aspergillus niger* Protein Data bank (PDB: 2GVY; red) and GH13_6 barley AMY1 (1P6W; green) supporting the closer relatedness of BaqA (novel GH13 subfamily) to GH13_1 (anticipated also from the evolutionary tree). (d) A maltose molecule (on the left) bound by Tyr382 and Trp385 (red) outside the active site in the *A. niger* α-amylase structure with corresponding residues in BaqA (blue) and barley AMY1 (green), as well as the 4^{I}, 4^{II}, 4^{III}-trithiomaltotetraoside (on the right) with Tyr380 (green) from the sugar tongs-like binding site of barley AMY1 without any correspondences in both BaqA and *A. niger* α-amylase.

CSR II) of the catalytic $(\beta/\alpha)_8$-barrel domain (Fig. 7) represent a sequence fingerprint of this new GH13 α-amylase subfamily. It is important to note that such two consecutive tryptophans implicated in sugar recognition and eventually also binding are not unique for the newly proposed GH13 subfamily. For example, in barley α-amylases, two tryptophans (Trp276 and Trp277, isozyme AMY2 numbering) were shown to form a well-defined secondary sugar-binding site (Gibson and Svensson 1987; Søgaard *et al.* 1993), and in mammalian counterparts, also two consecutive tryptophans (Trp58 and Trp59; human salivary α-amylase numbering) are important for sugar recognition (Ramasubbu *et al.* 1996; Gyemant *et al.* 2009). Inspection of the superimposed tertiary structures of barley α-amylase isozyme AMY2 (Kadziola *et al.* 1998) and human salivary α-amylase (Ramasubbu *et al.* 1996) with the predicted model of BaqA with focus on the above-mentioned tryptophan pairs confirmed that the two consecutive tryptophans are located in different parts of the structure of each of the three α-amylases (not shown). This observation supports the idea that Trp201 and Trp202 of BaqA may be the unique sequence-structural feature of the new GH13 α-amylase subfamily.

Mammalian α-amylases, moreover, together with some bacterial homologues, for example, that from *Pseudoalteromonas haloplanktis* (Aghajari *et al.* 2002), are activated by (or dependent on) a chloride ion (D'Amico *et al.* 2000). The BaqA sequence does not contain an arginine or lysine residue corresponding with the Arg300 of *P. haloplanktis* α-amylase found to be crucial for interaction with the chloride anion (Aghajari *et al.* 2002). In addition, the proposed GH13 subfamily represented by BaqA appears to be most closely related to the subfamily GH13_1 (fungal enzymes, e.g. Taka-amylase A), which is not known to be activated by (or dependent on) chloride ion (Matsuura *et al.* 1984; van der Kaaij *et al.* 2007). On the other hand, the chloride-dependent α-amylases (D'Amico *et al.* 2000; Cipolla *et al.* 2012) are members of the subfamilies (cf. Fig. 6) GH13_24 (mammals; vertebrates), GH13_15 (insects) and eventually GH13_32 (actinomycetes). The best studied bacterial chloride-dependent α-amylase from *P. haloplanktis* has not been classified into any GH13 subfamily as yet (http://www.cazy.org/), although it is evident that it is related to the large cluster of the three GH13 subfamilies 24, 15 and 32 (Da Lage *et al.* 2004).

The best α-amylase template for modelling was the Taka-amylase (Matsuura *et al.* 1984). No SBD, that is, a separate carbohydrate-binding molecule is seen (Fig. 8A), that usually is found in raw starch–degrading amylolytic enzymes (Machovic and Janecek 2006; Janecek *et al.* 2011). α-Amylases able to degrade raw starch without a separate SBD are rare; examples are barley isozyme AMY1 from plants (Tibbot *et al.* 2002; Robert *et al.*

2005) and the yeast α-amylase from *S. fibuligera* KZ (Hostinova *et al.* 2010). The two above-mentioned tryptophans (Trp210 and Trp202) of BaqA may contribute to its raw starch–degrading ability, that is, to act as binding residues enabling the stacking interactions with glucose molecules because their side chains are positioned outside the space occupied by the catalytic triad (Fig. 8B). A higher homology, expected also from the evolutionary tree (Fig. 6), is evident also from the structural comparison (Fig. 8C) of BaqA with GH13_1 *Aspergillus niger* α-amylase (root-mean-square deviation 1·32 Å for 420 corresponding C_α atoms) and GH13_6 barley AMY1 (1·83 Å for 271 C_α atoms). The *A. niger* α-amylase was chosen because it is 100% identical in sequence with TAKA-amylase and a maltose molecule bound outside the active site was found in its structure (Vujicic-Zagar and Dijkstra 2006). Based on the overlap, the two aromatic residues Tyr382 and Trp385 responsible for binding the maltose in the *A. niger* α-amylase structure have no proper (i.e. aromatic) counterparts in the BaqA model (Thr395 and Pro398, respectively) and the Tyr380 acting in the barley AMY1 sugar tongs-like binding site has no corresponding residue in BaqA at all (Fig. 8D). Concerning the starch granule binding surface site on catalytic domain A of the barley AMY1 (Trp278 and Trp279), which is also present in the isozyme AMY2 (Kadziola *et al.* 1998), there are no counterpart residues again in the corresponding region of the modelled BaqA structure (not shown) and the two BaqA signature trytophanes (Trp201 and Trp202) are located elsewhere in domain A. The results suggest that the amino acid residues responsible for raw starch binding in BaqA are probably unique for the new GH13 subfamily proposed in this study.

In conclusion, this is the first report of the amino acid sequence of a raw starch–degrading α-amylase from a soft coral associated *B. aquimaris* MKSC 6.2. The BaqA may form a new cluster of glycoside hydrolase subfamily GH13 together with several putative *Bacillus* α-amylases.

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